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The Synergistic Toxicity of Pesticide Mixtures: Implications for Risk Assessment and the Conservation of Endangered Pacific Salmon

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Abbreviations:

AChE – acetylcholinesterase

CB – carbamate insecticide

NOAA – National Oceanic and Atmospheric Administration

OP – organophosphate insecticide

U.S. EPA – U.S. Environmental Protection Agency

USGS – U.S. Geological Survey

Outline:

Abstract

Background: Mixtures of organophosphate and carbamate pesticides are commonly detected in freshwater habitats that support threatened and endangered species of Pacific salmon (*Oncorhynchus sp.*). These pesticides inhibit the activity of acetylcholinesterase (AChE), and thus have potential to interfere with behaviors that may be essential for salmon survival. While the effects of individual anticholinesterase insecticides on aquatic species have been studied for decades, the neurotoxicity of mixtures is still poorly understood.

Objectives: We assessed whether chemicals in a mixture act in isolation (resulting in additive AChE inhibition) or whether components interact to produce either antagonistic or synergistic toxicity.

Methods: We measured brain AChE inhibition in juvenile coho salmon (*O. kisutch*) exposed to sublethal concentrations of the organophosphates diazinon, malathion, and chlorpyrifos as well as the carbamates carbaryl and carbofuran. Concentrations of individual chemicals were normalized to their respective EC_{50} concentrations and collectively fit to a non-linear regression. This curve was used to determine whether toxicological responses to binary mixtures were additive, antagonistic, or synergistic.

Results: Addition and synergism were both observed, with a greater degree of synergism at higher exposure concentrations. Several combinations of organophosphates were lethal at concentrations that were sublethal in single chemical trials.

Conclusion: Single chemical risk assessments are likely to underestimate the impacts of these insecticides on salmon in river systems where mixtures occur. Moreover, mixtures of pesticides that have been commonly reported in salmon habitats may pose a more important challenge for species recovery than previously anticipated.

Introduction

Pesticides are chemical substances that are used to kill, repel, or regulate the growth of biological organisms. This diverse group includes insecticides, herbicides, fungicides, nematicides, acaricides, rodenticides, avicides, wood preservatives, and antifoulants. The U.S. Environmental Protection Agency (U.S. EPA) recently estimated that more than 1.2 billion pounds of pesticides are applied to crops, forests, residential areas, public lands, and aquatic areas in the United States each year (Kiely et al. 2004). The release of these chemicals into the environment creates a potential for unintended adverse health impacts to both humans and non-target wildlife.

Mixtures of pesticides are common in the human food supply (NRC 1993). Pesticide mixtures are also common in the aquatic environment, including lakes, river, streams and other surface waters that support aquatic life (Gilliom 2007). Assessing the cumulative toxicity of pesticides in mixtures has therefore been an enduring challenge for environmental health research (Monosson 2005) as well as ecotoxicology (Eggen et al. 2004) for the past several decades. In 1996 the United States Congress passed the Food Quality Protection Act (FQPA 1996), which directs the U.S. EPA to assess the human health risks from cumulative exposures to pesticides that share a common mechanism of action. Consideration of mixture toxicity is also required when pesticide tolerances are reassessed under the Federal Food, Drug, and Cosmetic Act (FFDCA 1938). At present, for aquatic species, there are no equivalent mandates for consideration of mixture toxicity under the Federal Insecticide Fungicide and Rodenticide Act (FIFRA 1972) or in the development of aquatic life criteria under the Clean Water Act 1972 (CWA 1972; Lydy et al. 2004).

The cumulative toxicological impacts of pesticide mixtures is of particular concern for salmon and steelhead populations that are currently listed as either threatened or endangered under the U.S. Endangered Species Act (ESA 1973). Many wild salmon stocks are in decline across much of the western U.S. (Nehlsen et al. 1991; NOAA Fisheries 2008). Past salmon population extinctions (Nehlsen et al. 1991) and current declines have been caused by decades of habitat degradation, overharvest, hydropower operation, and hatchery practices (NRC 1996). Major river systems that drain large agricultural and urban areas in California, Oregon, Washington, and Idaho provide freshwater habitat for ESA-listed salmon and steelhead (Figure 1). Extensive surface water monitoring for pesticides, as part of the U.S. Geological Survey's National Water Quality Assessment program (NAWQA), has shown that current-use pesticides are frequently detected in these salmon-supporting river systems (Table 1; see also more recent monitoring studies by Carpenter et al. 2008; Ecology 2008; USGS 2008). Furthermore, pesticides almost always occur in mixtures with other pesticides. Analysis of NAWQA monitoring data found that > 90% of water samples from urban, agricultural, and mixed-use streams contained two or more pesticides (Gilliom 2007). The toxicological effects of these mixtures on the health of salmon are largely unknown.

In the years since the enactment of the FQPA, the U.S. EPA has identified several classes of pesticides that share a common mode of action (USEPA 2002). Among these are the organophosphate (OP) and N-methyl carbamate (CB) insecticides. These two classes of chemicals inhibit the enzyme acetylcholinesterase (AChE), thereby interfering with cholinergic neurotransmission in both humans (Chambers 1992) and fish (Fulton and Key 2001). Because anticholinesterase agents share a common mode of toxic action, the National Academy of Sciences recommended a dose-additive approach to assessing risks to human infants and

children. Dose-addition (or, for waterborne exposures to fish, concentration-addition) assumes that the cumulative toxicity of the mixture can be estimated from the sum of the individual toxic potencies of each individual component chemical. This is how the U.S. EPA currently assesses the potential toxicity of mixtures of OP and CB insecticides in the context of the FQPA.

The assumption of dose- or concentration-addition for mixtures of anticholinesterase pesticides has also been extended to aquatic life (Junghans et al. 2006). In salmon, concentration-additive inhibition of brain AChE activity by mixtures of OPs and CBs was recently demonstrated *in vitro* (Scholz et al. 2006). However, the *in vivo* toxicity of anticholinesterase mixtures may deviate from concentration-addition if the individual chemicals in a mixture interact via toxicokinetic or toxicodynamic processes to produce either antagonistic or synergistic effects (Borgert et al. 2004). Each of these possible outcomes (antagonism, addition, or synergism) has potentially important implications for the current regulatory paradigm, wherein risks of pesticides to ESA-listed salmonids are assessed based primarily on responses to single active ingredients. To define the extent to which OP and CB insecticides in mixtures interact, we exposed juvenile coho salmon (*Oncorhynchus kisutch*) to all possible binary combinations of the OPs diazinon, malathion, and chlorpyrifos and the CBs carbaryl and carbofuran. The concentration-response curves for AChE inhibition by individual chemicals were used to statistically define concentration-addition (i.e., no interaction within a mixture).

Methods

Fish

Coho salmon eggs were obtained from the University of Washington hatchery (Seattle, WA) and raised at the Northwest Fisheries Science Center's hatchery (NWFSC, Seattle, WA).

Juveniles were maintained at the Washington State University Extension Campus (Puyallup, WA) for the duration of the study. Fish were held in recirculating tanks of dechlorinated municipal water (hatchery water; temperature 11-13 °C, pH 7.0-7.5, dissolved oxygen 90-100%, total hardness as $CaCO_3$ 110-120 mg/l, and alkalinity 74 mg/l) on a 12 hr light-dark schedule. Fish were fed commercial salmon pellets (Bio-Oregon, Warrenton, OR) daily. Fish used in experiments were 4-7 months old, with an average size (± SD) of 4.9 ± 1.0 cm and 1.3 ± 0.9 g. Experiments followed guidelines set by Washington State University's Institutional Animal Care and Use Committee (IACUC) for the humane treatment of fish to alleviate suffering exposures and dissections.

Pesticide exposures

Diazinon (#333-41-5; 98% pure), malathion (#121-75-5; 98% pure) chlorpyrifos (#2921-88-2; 98% pure), carbaryl (#63-25-2; 99% pure), and carbofuran (#1563-66-2; 99% pure) were purchased from Chem Service (West Chester, PA). Exposure concentrations used for both single pesticide and mixture exposures are shown in Table 2. Pesticide-containing stock solutions were prepared in methanol (or ethanol for chlorpyrifos) and added in 100 µl aliquots to 25 l of hatchery water in 30 l glass aquaria. Final carrier concentration in exposure tanks was ≤ 0.0004% of the total volume. For each treatment, 8 individual fish were exposed for 96 hrs on a 24 hr static renewal schedule. Animals were not fed during the exposure interval. Following exposures, fish were terminally anaesthetized by immersion in MS-222 (tricaine methanesulfonate, 5 g/l, Sigma, St. Louis, MO) until gill activity ceased. Brain tissues were removed, put into plastic microcentrifuge tubes, and kept on ice until being stored in a cryogenic (-80 °C) freezer for subsequent analyses of AChE enzymatic activity. In the three mixtures

exposures where mortality was observed, dead fish were removed after 24 hrs of exposure and processed for AChE analysis as described above.

Analytical chemistry

Water samples were collected in 500 ml amber glass bottles from exposure tanks immediately following pesticide addition. All analyses were conducted at Washington State University's Food and Environmental Quality Laboratory (Richland, WA) following existing U.S. EPA methods. Measured pesticide concentrations were generally between 80 and 120% of nominal concentrations (Table 3). For single chemical exposures, water samples collected at 96 hrs indicated only a modest loss (average 5 to 14 %) in pesticide concentration over the course of a 24 hr renewal interval. Accordingly, for subsequent exposures to mixtures, water samples were only collected at t = 0. Pesticide exposures throughout this paper are reported in terms of nominal concentrations, which in most cases will over-estimate actual exposure. Levels of chlorpyrifos in single pesticide exposures were not determined analytically because a recent study (Sandahl et al. 2005) characterized the concentration-response relationship between nominal (and measured) chlorpyrifos exposure and brain AChE inhibition in juvenile coho across a range of concentrations identical to the nominal chlorpyrifos exposures used here (0 – 2.5 μg/l). Thus, in the present study, AChE activity relative to published results (Sandahl et al. 2005) was used to confirm accurate dosing.

AChE enzyme assays

Determination of AChE activity followed the Ellman method (Ellman et al. 1960) as modified by Sandahl and Jenkins (Sandahl and Jenkins 2002). Briefly, whole brains were

homogenized at 50 mg/ml in 0.1 M sodium phosphate buffer (pH 8.0) with 0.1% Triton X-100. Homogenates were centrifuged, and 15 µl of the supernatant were combined with 685 µl of 10 mM phosphate buffered saline, 50 µl of 6 mM DTNB (5,5'-dithio-bis(2-nitrobenzioc acid)), and 30 µl of 75 mM acetylthiocholine iodide. All chemicals were from Sigma (St. Louis, MO). Triplicate 200 µl samples were transferred to a 96-well plate, and the change in absorbance (at 412 nm) was measured at 12 s intervals for 5 min at 25 °C on an Optimax plate reader (Molecular Devices, Sunnyvale, CA). AChE activity was quantified as mOD/min/g tissue and reported as a percentage of the baseline enzyme activity for fish exposed to carrier alone.

Statistics and data analysis

Statistical analyses were performed with either Prism 4.0 (GraphPad, San Diego, CA) or KaleidaGraph (Synergy Software, Reading, PA) software. Tests included non-linear regression to fit curves of AChE activities, one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test to establish differences between groups, and one-sample t-test with a Bonferroni correction to test for differences between means and predicted values. To allow for a Gaussian distribution of the error around the estimate of EC_{50}, the non-linear regression performed by Prism 4.0 uses log transformations of the concentrations and reports an estimate of the log transformation of EC_{50}.

Defining toxicological interactions between pesticides

Significant departures from additive toxicity were used to define antagonistic and synergistic interactions between pesticides in mixtures (Hertzberg and MacDonell 2002). Addition, in turn, describes an outcome of no interaction, where the predicted toxicity of the

mixture (as measured by brain AChE inhibition) is the sum of each chemical's predicted toxicity (toxic potential). The individual toxic potential for all five pesticides was determined empirically from the concentration-response relationship for AChE inhibition in single chemical trials. The concentration of each pesticide was normalized to the respective EC_{50} concentration (the concentration estimated to produce a 50% decrease in AChE activity relative to carrier controls) for that individual chemical. The EC_{50}-normalized data for all five pesticides were subsequently combined and fit with a single regression. A hypothetical example of a resulting curve, and its application to assessing mixture interaction, is shown in Figure 2A. For a mixture containing two pesticides at 0.1 and 0.3 EC_{50} units respectively, concentration-addition occurs if the cumulative AChE inhibition is equivalent to 0.4 EC_{50} units. This outcome would fall on the curve or within the 95% confidence interval for the regression. Results falling significantly above the curve (less than expected inhibition) would be antagonistic and results falling significantly below the curve (more than expected inhibition) would be synergistic. In this way, the curve fit to the data from single chemical trials was used as a basis for detecting interactions between OP and CB pesticides in mixtures.

Results

Single pesticides

Exposure to individual pesticides for 96 hr resulted in sublethal, concentration-dependent decreases in brain AChE activity among juvenile coho salmon. No mortality was observed at any of the single chemical exposure concentrations. There were no significant differences in baseline AChE activity between unexposed fish and those exposed to carrier alone (5 one-way ANOVAs, $p > 0.26$). Therefore, AChE activity in pesticide-exposed fish is expressed as a

percentage of carrier controls. The mean AChE activity (± SD) for all controls (n = 109 animals) was 121.49 ± 13.18 mOD/min, or 14.7 ±1.6 μmol/min/g. Concentration-response relationships were fit using a non-linear regression. The equation and resulting curve fit parameters are reported in Table 4. Despite the potential for variability from toxicokinetic differences in absorption, distribution, metabolism, and excretion, the slopes of the concentration-response curves were not significantly different (average = 0.96, F-test, $p = 0.1$). The relative potencies of the five chemicals did vary, with chlorpyrifos > carbofuran > malathion > diazinon > carbaryl (Table 2: EC_{50} values for AChE inhibition). The EC_{50}-normalized data for all five pesticides was then fit with a single non-linear regression ($r^2 = 0.94$; Figure 2B). As noted above, this curve was used as a basis to quantitatively determine whether specific binary combinations of pesticides produce interactive (i.e., antagonistic or synergistic) toxicity.

Pesticide mixtures

Based on a default assumption of dose-addition, the five pesticides were combined in all possible pairings to yield predicted AChE inhibitions of 10%, 29%, and 50% in the brains of exposed coho salmon. As determined by the regression in Figure 2B, these levels of enzyme inhibition would result from exposure to 0.1, 0.4, and 1.0 EC_{50} units, respectively. All binary pesticide combinations produced toxicity that was either additive or synergistic, with the frequency of synergism increasing at higher exposure concentrations (Figure 2C). In all cases, the joint toxicity from the paired exposures resulted in AChE activities that were significantly lower than carrier controls (one-way ANOVA; $p < 0.05$).

The degree of AChE inhibition in response to specific combinations of OPs and CBs is shown in Figure 3. For each of the three EC_{50} units (0.1, 0.4, and 1.0), the measured values for

AChE activity are plotted as bars that originate from a horizontal line indicating a (non-interactive) dose-additive response. All combinations of insecticides at each of the three sets of concentrations deflected downwards, indicating a tendency towards synergism. Most pesticide pairs yielded rates of enzyme activity that were significantly lower than would be expected based on concentration-addition (t-test with Bonferroni correction, $p < 0.005$). The number of combinations that were statistically synergistic (asterisks in Figure 3; 20 of 30 pairings overall) increased with increasing exposure concentrations. Additionally, pairings of two OPs produced a greater degree of synergism than mixtures containing one or two CBs. This was particularly true for mixtures containing malathion together with either diazinon or chlorpyrifos. At the highest exposure concentration (1.0 EC_{50}), the toxicity of every insecticide mixture was synergistic.

Coho exposed to combinations of diazinon and malathion (1.0 and 0.4 EC_{50}) as well as chlorpyrifos and malathion (1.0 EC_{50}) had the lowest measured AChE activities. Many fish species die following high rates of acute brain AChE inhibition (> 70-90%; Fulton and Key 2001). As expected from these previous studies, 100% mortality was observed within the first 24 hr among coho exposed to the above pesticide combinations. Fish exposed to these OP mixtures also showed qualitative signs of anticholinesterase toxicity, including loss of equilibrium, rapid gilling, altered startle response, and increased mucus production. Although no mortality was observed among coho exposed to the lowest combinations of diazinon and malathion (0.1 EC_{50}), all of the fish in this treatment group displayed overt symptoms of sublethal cholinergic poisoning by the end of the 96 hr exposure interval. Therefore, biochemical indicators of synergism (greater-than-additive AChE inhibition) were consistent

with classical signs of anticholinesterase intoxication and death for salmon exposed to mixtures of OPs.

Discussion

We have shown that *in vivo* exposures to binary mixtures of OP and CB pesticides produced additive or synergistic AChE inhibition in the brains of juvenile coho salmon. The statistical departure from dose-addition occurred for several chemical combinations at each of the three relative exposure concentrations, with a trend towards a higher incidence of synergism at the higher exposures. Where the degree of synergism was severe (e.g., for pairings of diazinon and malathion), enhanced AChE inhibition (i.e., > 90%) corresponded to overt signs of anticholinesterase intoxication and death. This result is consistent with previous (single chemical) OP and CB toxicity studies in other fish species (reviewed by Fulton and Key 2001). At present, diazinon, chlorpyrifos, malathion, carbaryl, and carbofuran are some of the most extensively used insecticides in California and the Pacific Northwest (California DPR 2008). The frequency with which these chemicals are detected in some salmon habitats (Table 1), and their combinatorial toxicity to juvenile salmon when they occur as mixtures, suggest they may be limiting the recovery of several threatened and endangered populations.

The OPs (oxon metabolites) and CBs examined in this study do not interact *in vitro*, where their combinatorial inhibition of salmon AChE can be explained by simple concentration-addition (Scholz et al. 2006). The departure from concentration-addition for some pesticide pairings *in vivo* is consistent with OPs and CBs acting on other biochemical targets. Although more work is needed to identify these targets, carboxylesterases (CaE) are candidate enzymes that may underlie the chemical interactions observed in this study. CaEs play an important role

in the detoxification of many pesticides, including the OP and CB insecticides, via hydrolysis (Jokanovic 2001; Wheelock et al. 2005a). CaEs may also functionally protect AChE from insecticide toxicity by direct binding and sequestration, thereby preventing or delaying interaction between the insecticide and AChE (Jokanovic 2001; Maxwell 1992a). Mammalian studies spanning several decades have shown that anticholinesterase toxicity increases when CaE enzyme activity is inhibited (Casida et al. 1963; Jokanovic 2001; Maxwell 1992b; Su et al. 1971). Although few studies are documented in fish, exposures to OPs and CBs have been found to reduce liver CaE activity in salmonids (Ferrari et al. 2007; Wheelock et al. 2005b), with the OP chlorpyrifos acting as a more potent inhibitor of CaE activity than AChE activity (Wheelock et al. 2005b). In another aquatic species (*Daphnia magna*), pharmacological inhibition of CaE significantly enhanced the toxicity of chlorpyrifos, malathion, and carbofuran (Barata et al. 2004). Thus, while other biochemical targets may be involved in OP and CB synergism (Casida and Quistad 2004), future mechanistic studies should give particular consideration to the role of CaEs in the pesticide interactions observed in this study.

To identify interactions between pesticides in mixtures, it was first necessary to normalize each concentration-response curve using the calculated EC_{50} concentration for that individual chemical. For all five insecticides, the concentrations that produce 50% brain AChE inhibition in salmon (Table 2) are approximately 10 to 1,000-fold higher than the levels typically detected in surface water monitoring investigations (Hoffman et al. 2000). However, we show here that many insecticide combinations produce additive toxicity at low, environmentally relevant concentrations (0.1 EC50; upper panel in Figure 3). Moreover, certain combinations showed a clear pattern of synergism even at these relatively low levels. For example, diazinon and chlorpyrifos were synergistic when combined at 7.3 μg/L and 0.1 μg/L, respectively.

Surface water monitoring in the San Joaquin basin (Dubrovsky et al. 1998) reported diazinon concentrations as high as 6.0 μg/L and chlorpyrifos levels up to 0.5 μg/L. The pairing of diazinon (7.3 μg/L) with malathion (3.7 μg/L) produced severe (> 90%) AChE inhibition as well as classical signs of anticholinesterase poisoning. Thus, for some chemical combinations, synergism is likely to occur at exposure concentrations that are below the lowest levels used in the present study. Although more work is needed to determine the lower bounds for pesticide interactions, this study indicates that synergism is likely to occur at concentrations that have been directly measured in habitats supporting threatened and endangered salmonids.

In quantitative terms, we have shown that an *in vivo* screen for interactions between anticholinesterase insecticides is tractable in juvenile salmon. Although we examined only five pesticides, it would be straightforward to establish concentration-response relationships for AChE inhibition for the remaining OPs and CBs in current use. Given default assumptions of common mode of action and concentration-addition (Lambert and Lipscomb 2007), the relative potency of each insecticide could then be used to estimate the joint toxicity of chemicals in a mixture using a conventional toxic unit approach (Junghans et al. 2006). Widely used insecticides and those with a relatively high toxic potency (e.g., the OP azinphos-methyl) could also be screened for interactions with other insecticides at low, environmentally realistic exposure concentrations. Where synergism occurs, additional safety factors could then be assigned to protect the health of threatened and endangered salmon. With the exception of safety factors for synergism, this process is similar to how the U.S. EPA is evaluating the human health risks of OP and CB mixtures as mandated by the 1996 Food Quality Protection Act (FQPA 1996).

Although habitat degradation is generally accepted to be a major causal factor in salmon

declines (NRC 1996), the specific contributions of current use pesticides to the decline of salmon populations are not well understood. One key challenge to understanding this relationship is linking pesticide effects on individual fish to the intrinsic productivity of populations. Recent data by Sandahl et al. (Sandahl et al. 2005) began to address this challenge by showing that exposures to low, environmentally realistic concentrations of one of the pesticides used in this study, chlorpyrifos, produced reductions in AChE activity that were closely correlated to reductions in swimming speed and feeding rates. Reductions in feeding are likely to lead to reductions in the size of exposed salmon at the time of their seaward migration, an endpoint that has been shown to be an important determinant of individual salmon survival (Higgs et al. 1995; Zabel and Achord 2004). By reducing survival rates, sublethal inhibition of AChE in juvenile salmon could potentially reduce the intrinsic productivity of salmon populations. Because mixtures of OPs and CBs produce dose-additive or synergistic AChE inhibition, they could magnify these population-scale effects.

The link to populations is important because most of the ongoing recovery planning for ESA-listed salmon is focused at this biological scale (Ruckelshaus et al. 2002). Although many salmon habitats are impacted by agrochemicals and urban runoff, restoration priorities are usually developed without the specific inclusion of toxics in quantitative analyses of limiting factors (Bartz et al. 2006; Burnett et al. 2007; Hoekstra et al. 2007; Scheuerell et al. 2006). In the larger context of salmon conservation, a future priority will be to establish a quantitative connection between the mixture toxicity observed in this study and higher biological scales via effects on growth and survival. This connection will help to bridge the disciplines of ecotoxicology and conservation biology (Hansen and Johnson 1999) in their common goal of guiding the recovery of threatened and endangered species.

In conclusion, these results have important implications for ecological risk assessments, particularly those that focus on the toxicity of individual chemicals as the basis for estimating impacts to imperiled aquatic species. Although the importance of multiple stressors is widely recognized in aquatic ecotoxicology (Eggen et al. 2004), pesticide mixtures continue to pose major challenges for natural resource agencies (Gilliom 2007; Lydy et al. 2004). These challenges include the data gaps that exist for many individual chemicals, experimental design difficulties (e.g., near-insurmountable factorial complexity for large numbers of chemicals), poorly understood pathways for chemical interaction, potential differences in response among species, and the need for more sophisticated statistical tools for analyzing complex data. Salmon exposed to mixtures containing some of the most intensively used insecticides in the western United States showed either concentration-additive or synergistic neurotoxicity, as well as unpredicted mortality. This implies that single chemical assessments will systematically underestimate actual risks to ESA-listed species in salmon-supporting watersheds where mixtures of OPs and CBs occur.

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Table 1. Frequency of insecticide detections by the U.S. Geological Survey in surface waters of National Water Quality Assessment (NAWQA) basins in the western US.

	Frequency of Insecticide Detection (% of samples)				
NAWQA Basin	Diazinon	Malathion	Chlorpyrifos	Carbaryl	Carbofuran
Puget Sound (Ebbert et al. 2000)	48%	D	3%	D	D
Columbia Plateau (Williamson et al. 1998)	4%	2%	9%	6%	5%
Yakima River (Fuhrer et al. 2004)	18%	D	D	90%	ND
Willamette (Wentz et al. 1998)	35%	5%	21%	18%	29%
Sacramento River (Domagalski et al. 2000)	75%	33%	38%	60%	36%
San Joaquin (Dubrovsky et al. 1998)	71%	8%	52%	25%	5%

D = detected, frequency not reported

ND = not detected

Table 2. Nominal concentrations used in both single insecticide and mixtures exposures. EC_{50} values were calculated using non-linear regressions. For individual chemicals, salmon were exposed to n = 4-7 concentrations within the indicated range. Values are presented as μg/l.

	Single exposures		Mixture exposures		
Insecticide	Concentration	1.0	0.5	0.2	0.05
	Range	EC_{50}	EC_{50}	EC_{50}	EC_{50}
Diazinon	1.0-500	145.0	72.5	29.0	7.3
Malathion	0.5-100	74.5	37.3	14.9	3.7
Chlorpyrifos	0.6-2.5	2.0	1.0	0.4	0.1
Carbaryl	1.0-150	145.8	72.9	29.2	7.3
Carbofuran	1.0-225	58.4	29.2	11.7	2.9

Table 3. Chemical analysis of insecticide concentrations from both single insecticide and mixture exposures. Values are average percent recovery (relative to nominal concentrations) ± one standard deviation.

	Single	Mixtures		
Insecticide		1.0 EC_{50}	0.4 EC_{50}	0.1 EC_{50}
Diazinon	88 ± 18	63 ± 5	97 ± 11	106 ± 28
Malathion	89 ± 26	44 ± 27	91 ± 56	70 ± 27
Chlorpyrifos	NA	72 ± 7	79 ± 17	121 ± 15
Carbaryl	113 ± 21	118 ± 33	112 ± 9	108 ± 9
Carbofuran	115 ± 32	112 ± 12	130 ± 15	105 ± 6

NA = not analyzed

Table 4. Parameters of the concentration-response curves following *in vivo* exposures to individual insecticides. SE denotes standard error of the non-linear regression. EC_{50} values are presented as µg/l. Curves were produced from the non-linear regression equation $y = (100)/(1+x^{slope})$.

Insecticide	Log EC_{50} (± SE)	R^2	Slope (± SE)
Diazinon	2.2 ± 0.1	0.95	-0.79 ± 0.15
Malathion	1.90 ± 0.05	0.97	-1.32 ± 0.20
Chlorpyrifos	0.30 ± 0.02	0.98	-1.50 ± 0.17
Carbaryl	2.2 ± 0.1	0.95	-0.81 ± 0.16
Carbofuran	1.80 ± 0.09	0.95	-0.82 ± 0.15

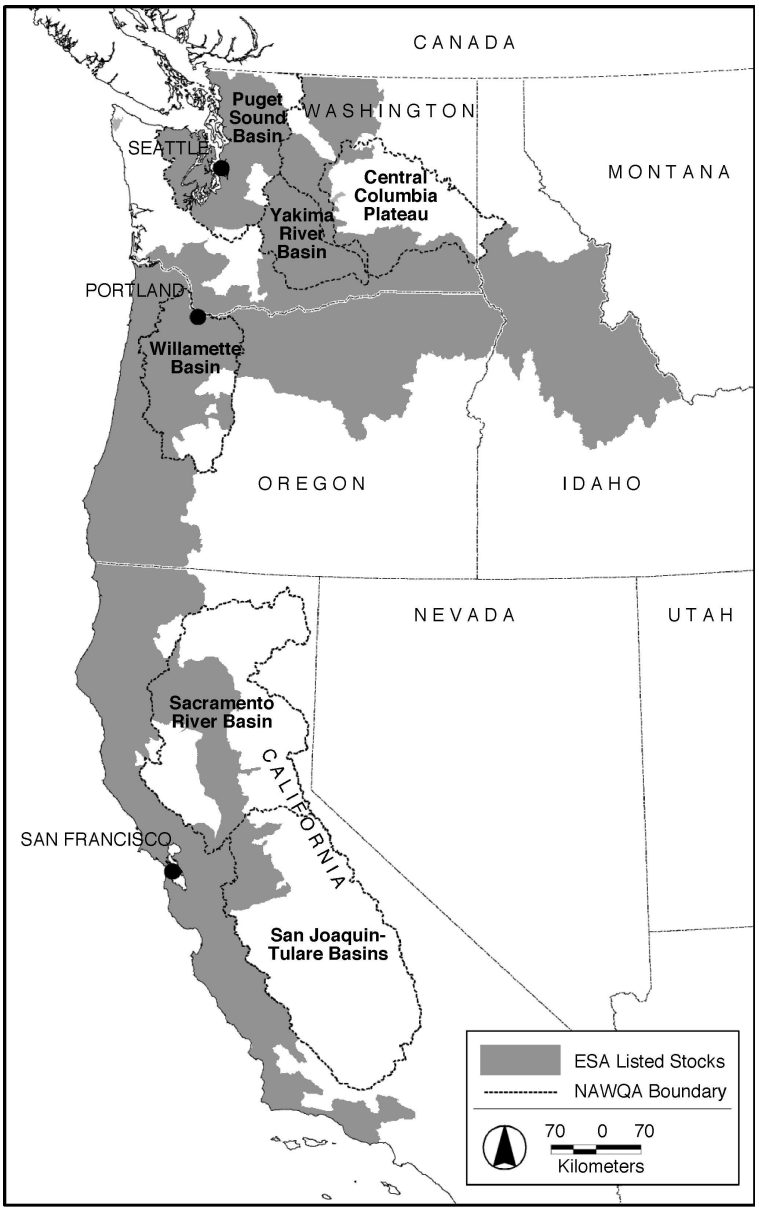
Figure Legends

Figure 1. The geographical distribution of threatened and endangered salmon in the western US overlaps with study units from the US Geological Survey's National Water Quality Assessment (NAWQA) program. Dashed lines mark the boundaries of NAWQA study areas where pesticide concentrations have been measured in surface waters. Gray shaded areas show the freshwater range of ESA-listed salmon populations.

Figure 2. Binary pesticide mixtures cause additive or synergistic acetylcholinesterase (AChE) inhibition. A) Hypothetical plot describing the three possible toxicological responses after exposure to a binary mixture of anticholinesterase pesticides. The curve represents a single regression fit to the EC_{50}-normalized data from single pesticide exposures. B) Plot of the concentration-response data from five single pesticide exposures following normalization to their respective EC_{50} concentrations and collectively fitting with a non-linear regression. This curve was used to evaluate the toxicological response of subsequent binary mixtures (panel C). Values are mean ± one standard error (n = 8). C) Plot of the brain AChE activities of fish exposed to the five pesticides in all possible binary combinations. Based on a default assumption of concentration-addition, the pairings were predicted to yield AChE inhibitions of 10% (0.1 EC50), 29% (0.5 EC50) and 50% (1.0 EC50). Values are mean and standard error (n=8), and the dashed lines indicate the 95% prediction band.

Figure 3. Plot highlighting differences in the toxicological response to binary combinations of organophosphate (OP) and carbamate (CB) pesticides. OP-OP pairings tended to be more synergistic than other pairings, producing 100% mortality (denoted by M) at concentrations that

were sublethal in single pesticide exposures. The number of combinations that were statistically synergistic (t-test with Bonferroni correction, denoted by asterisks) increased with increasing exposure concentrations. Bars are mean values (n = 8), and error bars show the 95% confidence intervals of the mean.



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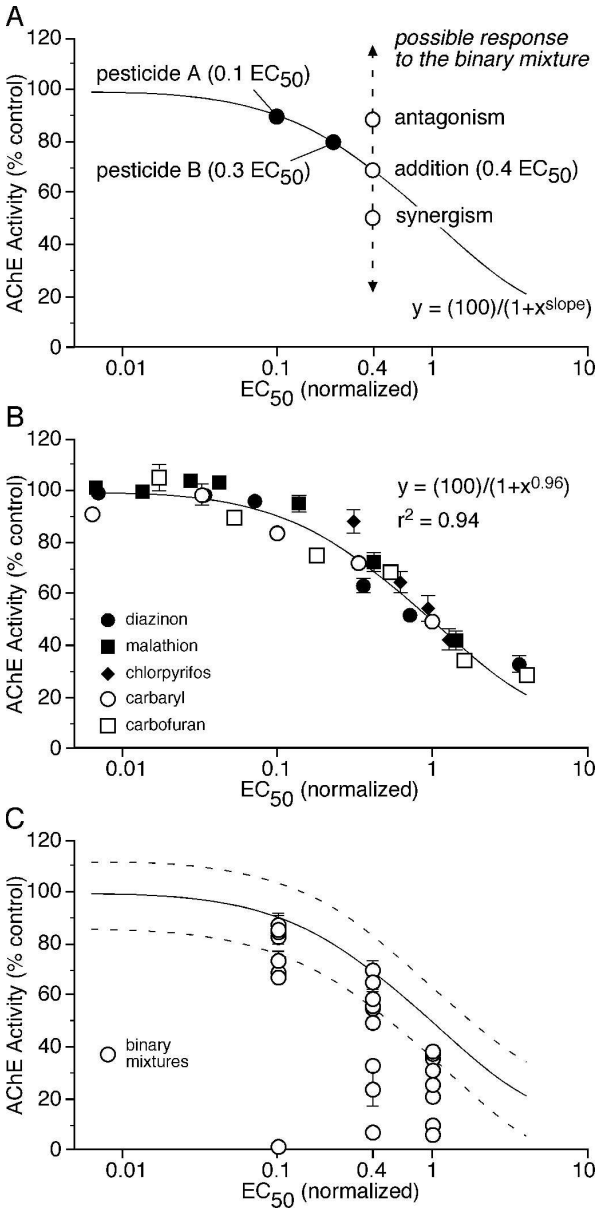

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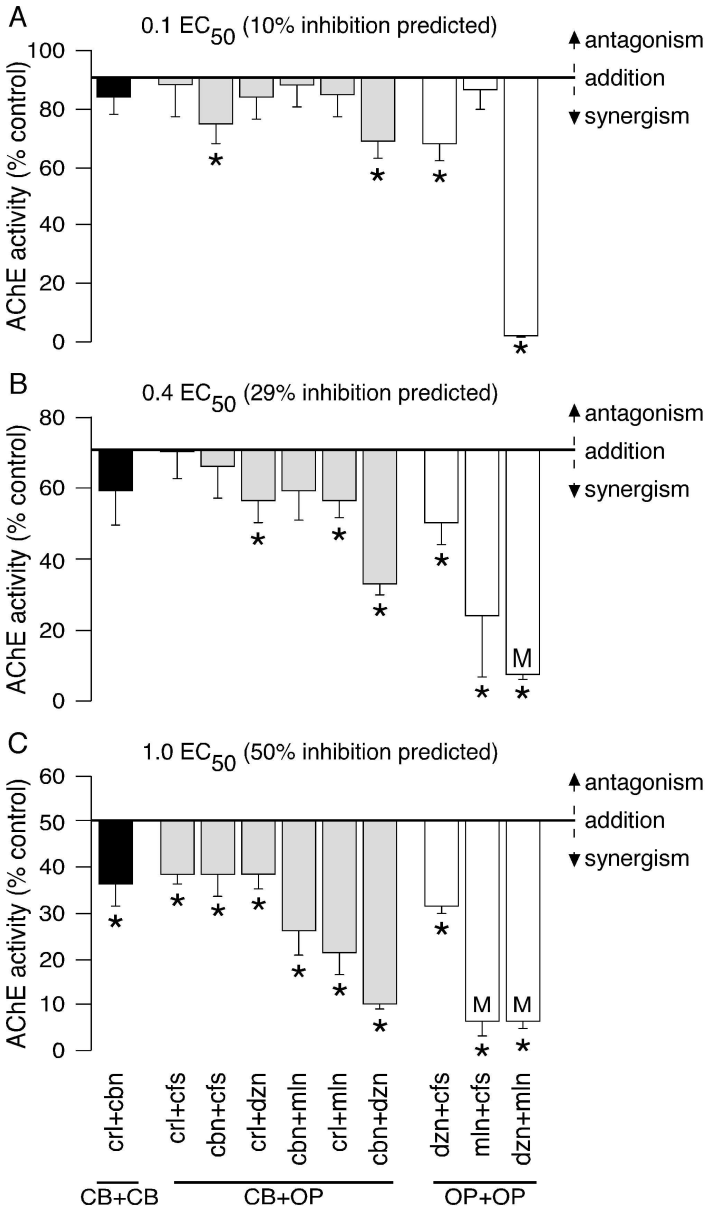

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